The statutory-type double patenting rejection of Claims 1-4, 6-10, 12-13, 16 and 22 over the specified claims of co-pending Serial No. 08/321,552 is maintained. This rejection is provisional because the conflicting claims in the co-pending case have not been patented. Applicant requests that this rejection be held in abeyance until such time as patentable subject matter is found in either case.

The obviousness-type double patenting rejection of Claims 5, 11, 14-15 and 17-21 over the specified co-pending claims of Serial No. 08/321,552 is also maintained. This is a provisional rejection since the conflicting claims have not, in fact, been patented. Applicant also requests that this rejection be held in abeyance until such time as patentable subject matter is found in either case.

Claims 1-21 are rejected under 35 U.S.C. § 103 as being unpatentable over Wu *et al.* in view of Kornguth *et al.*.

The present invention teaches compositions and methods using delivery vehicles which can specifically deliver physiological agents such as contrast agents and therapeutic agents. The delivery vehicles generally comprise four elements: (1) a first polymeric molecule having a net positive or negative charge; (2) a second polymeric molecule having a net charge opposite to the first polymeric molecule; (3) a cell targeting moiety attached to the second polymeric molecule; and (4) a physiological agent attached to either the first or second polymeric molecule (or a third polymeric molecule similar to the second polymeric molecule). The physiological agent can be a therapeutic agent, such as a drug, hormone, enzyme, protein or peptide, anti-cancer agent, etc., or a contrast agent, such as magnetic resonance imaging contrast agents, radioisotope contrast agents, gamma emitter contrast agents, positron emitter contrast agents, beta emitter contrast agents and optical contrast agents, including fluorescent contrast agents. The applicants submit that none of the prior art references, taken alone or in combination, teaches or suggests the present invention.

Wu *et al.* teaches the use of complexes with three components: (1) a DNA molecule; (2) a polycation; and (3) asialoglycoprotein molecules, which are used to target the complexes

to hepatocytes. As acknowledged by the Examiner, Wu *et al.* does not teach or suggest the addition of a physiological agent such as a therapeutic agent or contrast agent.

Kornguth *et al.* is directed to methods and compositions for imaging tumors which have a net positive charge and thus will preferentially bind to tumors which have a net negative charge. Kornguth *et al.* teaches the use of polylysine linked to a metal chelator, DTPA, (a "linking molecule") which then will bind a metal which can be used either for imaging (*i.e.*, paramagnetic metals such as Gd or Mn) or as a chemotherapeutic agent (*i.e.*, radioisotopes such as gamma or beta emitters). Kornguth *et al.* does not teach or suggest the use of a second polymer, nor does Kornguth teach or suggest the use of a cell targeting moiety.

The Examiner's position appears to be that since Wu can add asiaglycoprotein to polylysine, and Kornguth can add MRI contrast agents to polylysine, that there was some motivation to combine the two. The applicants respectfully disagree.

As stated in M.P.E.P. §2142, a *prima facie* case of obviousness requires three basic criteria to be met. First, there must be some suggestion or motivation to combine the reference teachings. Second, there must be a reasonable expectation of success. Finally, the references, taken alone or in combination, must teach or suggest all the claim limitations.

None of the references, taken alone or in combination, provide any motivation or suggestion to combine the references and practice the claimed invention. Wu *et al.* does not suggest using an MRI contrast agent. Kornguth *et al.* does not teach or suggest using a targeting moiety, since they rely on electrostatic interaction to provide selective binding.

In fact, Kornguth *et al.* teaches away from the claimed combination in several ways. First of all, the addition of a nucleic acid which has a high net negative charge to the polylysine would substantially decrease or eliminate the net positive charge of the polylysine, thus decreasing or eliminating the "targeting" function of the polylysine. Furthermore, Kornguth *et al.* does not teach or suggest the use of cell targeting moieties. Kornguth *et al.* relies on charge differences to effect targeting. In fact, adding the asialoglycoprotein from Wu *et al.* to the

Kornguth compositions could result in a loss of targeting in Kornguth, since the complexes would also be taken up by hepatocytes. This is very significant. As outlined in M.P.E.P. §2143.01:

> If a proposed modification would render the prior art invention being modified unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification.

See *In re Gordon*, 221 USPQ 1125 (Fed. Cir. 1984).

Furthermore, this modification would change the principle of operation of Kornguth *et al.* As stated in M.P.E.P. §2143.01:

> If the proposed modification or combination of the prior art would change the principle of operation of the prior art invention being modified, then the teachings of the references are not sufficient to render the claims *prima facie* obvious.

In re Ratti, 123 USPQ 349 (CCPA 1959).

Finally, the Examiner is respectfully reminded that a reference which leads one of ordinary skill in the art away from the claimed invention cannot render the invention obvious. See *Dow Chemical v. American Cyanamid Co.*, 2 USPQ2d 1350 (Fed. Cir. 1987).

In conclusion, neither reference, taken alone or in combination, provides the required motivation to combine the references. As acknowledged by the Examiner in the office action dated October 24, 1996, page 4, Wu *et al.* does not disclose the inclusion of an MRI agent. As argued above, Kornguth *et al.* does not provide the required motivation to combine the references, as (a) the combination would render the Kornguth system unsatisfactory for its intended purpose, (b) the combination would alter the mechanism by which the Kornguth *et al.* system functions, and (c) it actually teaches away from the combination in that a loss of targeting could occur.

Finally, the applicants respectfully remind the Examiner that the Supreme Court of the United States has stated that "such secondary considerations as commercial success, long felt but unsolved needs, failure of others, *etc.*, might be utilized to give light to the

circumstances surrounding the origins of the subject matter sought to be patented." *Graham v. John Deere Co.*, 148 USPQ 459 (1966).

The Federal Circuit has emphatically and repeatedly held that objective evidence of nonobviousness must be taken into account always and not just when the decision maker is in doubt: "objective evidence such as commercial success, failure of others, long-felt need, and unexpected results must be considered before a conclusion on obviousness is reached" (*Hybridtech Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81 (Fed. Cir. 1986). See also *Bausch & Lomb, Inc. v. Barnes Hindes, Inc.*, 230 USPQ 416 (Fed. Cir. 1986); *Jones v. Hardy*, 220 USPQ 1021 (Fed. Cir. 1984)).

In this case, the complexes of the invention show a surprising and unexpected benefit over the complexes of the prior art. As shown in the paper resulting from this work, Kayyem *et al.*, *Current Biol. 2*:615-620 (1995), a copy of which is enclosed as Exhibit A, the complexes comprising the four components (polycation, polyanion (DNA), transferrin targeting moieties and MRI contrast agent) showed a two-fold increase in the level of gene expression as compared to the three component system (polycation, polyanion, and transferrin); see Figure 3. The three component system is similar to the composition of Wu *et al.*; thus, the present invention shows a surprising and unexpected result: "It was surprising that the level of gene expression was increased approximately two-fold over that obtained using particles lacking Gd-DTPA-PDL" (see page 617, column 1, last paragraph).

Accordingly, the compositions of the present invention provide unexpected results over what could have been reasonably predicted by the prior art. See *In re O'Farrell*, 7 USPQ2d 1673 (Fed. Cir. 1988).

An additional factor to be evaluated in a "secondary considerations" analysis is the taking of licenses under the patent or technology. See *In re Sernaker*, 217 USPQ 1 (Fed.Cir. 1983). As shown in the accompanying declaration of Dr. Gary Blackburn, Vice President of Research, submitted pursuant to 37 C.F.R. §1.132, Clinical Micro Sensors has taken an exclusive, world-wide license to this technology.

Finally, the applicants point out that others in this field recognize that Dr. Meade's approach is a valuable contribution to the area of imaging and transfection. A paper published in 1997, nearly 3 years after the invention, is also directed to simultaneous contrast enhancement and transfection, and recognizes the work of Dr. Meade:

> Visualization of polynucleotide localization within lymph nodes by gadolinium contrast enhancement would be a rapid, noninvasive means of monitoring treatment, were the same agent also capable of simultaneously serving as the gene transfection agent. A report by Meade *et al.* on the cotransport of DNA and DTPA-Gd using polylysine in cell culture experimentation supports the feasibility of this approach.

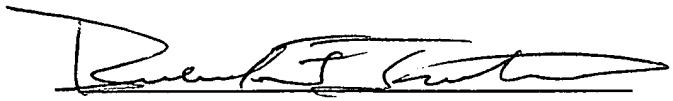
See page 3992, second column, first full sentence, of Wisner *et al.*, *J. Med. Chem* 40:3992-3996 (1997), a copy of which is enclosed as Exhibit B. Thus, others of skill in the art are working towards this same goal, and recognize Dr. Meade's contributions in this area.

Accordingly, the applicants submit that these secondary considerations indicate that the complexes of the invention are not obvious.

Based on the foregoing, it is submitted that the claims as pending in the case are patentable over the prior art of record.

Respectfully submitted,

FLEHR HOHBACH TEST
ALBRITTON HERBERT LLP



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Receptor-targeted co-transport of DNA and magnetic resonance contrast agents

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Background: Ligand molecules conjugated to polylysine can be electrostatically bound to DNA and can bind receptors or antigens on the surface of cells, delivering the DNA into specific cells and tissues. Several researchers have used this approach to generate non-viral vehicles for the efficient delivery of DNA to specific cells. We have attempted to adopt this general approach to the cell-specific delivery of magnetic contrast agents for use in magnetic resonance imaging (MRI).

Results: We have synthesized a new class of agents capable of both transfecting genes into cells and enhancing the contrast of the targeted cells for MRI. DNA is used both to encode a marker gene and as a molecular scaffold, which electrostatically binds polylysine conjugated to transferrin, an iron uptake protein, and polylysine modified with gadolinium chelated to diethylenetriaminepetaacetic acid. When cells displaying the transferrin receptor are treated with these particles, high levels of gene expression are observed, higher than with control particles composed only of transferrin, polylysine and DNA. The treated cells show specific MRI contrast enhancement, which did not require expression of the marker gene.

Conclusions: The development of this class of particles permits the use of novel protocols by which genes for genetic therapy and agents for MRI contrast are co-transported. These protocols may allow non-invasive MRI monitoring of DNA delivery for gene therapy in real time.

Chemistry & Biology September 1995, 2:615–620

Key words: contrast agent, gene delivery, MRI, polylysine, transferrin

Introduction

Magnetic resonance imaging (MRI) has become a powerful tool in clinical and research settings because it is non-invasive and yields an accurate volume rendering of a specimen. Using magnetic field gradients and selective radio frequency (RF) pulses, a one-, two- or three-dimensional image of the specimen is obtained. Typically, the image is based upon the NMR signal from the protons of water where the signal intensity in a given volume element (voxel) is a function of the water concentration and the relaxation times of the spins within the voxel (T_1 and T_2). Local variations in these three parameters provide the vivid contrast observed in MRI images [1]. The relaxation times can be strongly affected by MRI contrast agents, permitting specific fluid compartments or cells to be selectively followed. Unlike light-microscope imaging techniques that are based on the use of organic dyes or fluorochromes, neither MRI nor MRI contrast agents produce toxic by-products from photo-bleaching. Furthermore, MRI is not limited by light scattering or other optical aberrations, permitting high field strength instruments (>7 Tesla) to generate high resolution (10–15 μm) images of developing insects, fish, amphibia and mammals [2].

A major limitation on the use of MRI in basic research settings has been the invasive manipulations and/or microinjections required to label cells or tissues. In clinical settings, limitations resulting from similar constraints together with the potential for using MRI contrast agents to target specific tissues or tumors *in vivo*, have stimulated the syntheses of new paramagnetic agents covalently attached to biological macromolecules [3]. To significantly enhance the observed contrast, however, antibodies or other targeting molecules must bind very large numbers of paramagnetic complexes [4]. Such high ratios of contrast agent to biomolecule cripple the targeting molecule due to ionic and steric effects. Therefore, much of the current research in this field has focused on the use of liposomes as carriers of high concentrations of contrast agents [5], iron oxide particles as high signal strength T_2 contrast agents [6], and more recently, highly modified starburst dendrimers of paramagnetic agents [7].

Here we report a different strategy, based on methods currently under development for the delivery of nucleic acids to specific cells, to deliver both exogenous genes and large numbers of MRI contrast agents to cells. Polypeptides composed exclusively of lysine residues bind electrostatically to DNA and cause it to condense by neutralizing the negative charge. Ligand molecules conjugated to polylysine can thus be bound to DNA. Binding of the ligand molecule within the DNA–ligand complex to receptors or antigens on the cell surface delivers the complex with high efficiency into specific cells and tissues [8–15]. In our modification of this scheme, paramagnetic contrast agents are covalently attached to polylysine for incorporation into a DNA–polycation complex (Fig. 1). For our first test case, we used ternary particles containing the following components: i) DNA encoding the firefly luciferase reporter gene, ii) poly-L-lysine attached to human transferrin, a

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EXHIBIT A

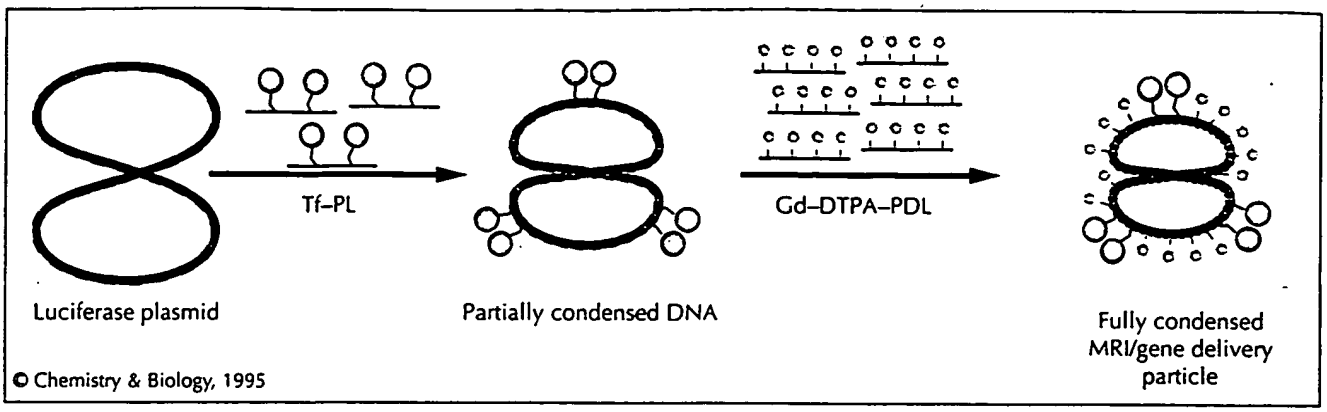

Fig. 1. Summary of the formation of ternary complexes for receptor-mediated uptake of DNA and MRI contrast agents. DNA is partially condensed by the addition of sub-optimal quantities of transferrin–polylysine (Tf–PL). Full condensation to electro-neutrality follows the addition of Gd–DTPA–PDL, producing particles with high transfection efficiency and MRI contrast enhancement.

protein involved in iron uptake, and iii) poly-D-lysine (PDL) attached to varying numbers of the paramagnetic contrast agent, gadolinium diethylenetriaminepetaacetic acid (Gd–DTPA). Uptake of one of these particles by cells expressing the transferrin receptor results in delivery of ~1500 Gd ions per cell, achieving the high ratio of contrast agent per biomolecule required for *in vivo* labeling. After incubation of human T-cell leukemia derived K562 cells with the ternary complexes, detectable MRI contrast enhancement is observed at nearly single-cell resolution. Moreover, the level of gene expression observed in these cells is higher than the levels observed using control particles that do not contain the MRI contrast agent.

Results and discussion

Synthesis and characterization of Gd–DTPA conjugates of PDL

We prepared a series of DTPA-modified PDL derivatives with varying numbers of labeled sites. Simply dissolving PDL in aqueous solutions at high pH, followed by addition of DTPA anhydride, invariably leads to a large distribution of products. At concentrations above 100 μM PDL, the major product isolated from this reaction is a crosslinked material of high molecular weight, as revealed by fast protein liquid chromatography (FPLC). By maintaining the PDL concentration below 100 μM and controlling the rate of anhydride addition, we have modified the PDL used in these experiments with as few as 7 and as many as 60 DTPA chelates with no evidence of crosslinking.

Fluorescence titration experiments were performed to assess the degree of substitution on the 180 residue PDL backbone resulting from various molar input ratios of DTPA and PDL. Europium chelates of DTPA form readily at room temperature and show characteristic and distinct fluorescence with large Stoke's shifts and long fluorescent lifetimes. Titration of the DTPA–PDL conjugates with $EuCl_3$ produced increasing fluorescence intensities followed by inflections in the curves corresponding to complete saturation of the DTPA sites [16]. The ratios of DTPA to PDL determined by fluorescence titration of the various conjugates is shown in Table 1 along with the calculated number of unmodified (and thus positively charged) lysines per DTPA site.

Previous studies on the interaction between DNA and polylysine have suggested that polymers containing seven or more lysine monomers readily form neutral particles with DNA, whereas shorter polymers do not [17]. Therefore, we anticipated that PDL modified with 10 and 22 Gd chelates per PDL chain (16 lysines and 6.7 lysines per Gd chelate, respectively) would interact readily with DNA, whereas PDL modified with 42 and 51 Gd chelates, which have shorter stretches of unmodified lysine residues, would not.

To rapidly test this hypothesis, the effect of PDL conjugates on the secondary structure of DNA in solution was indirectly monitored using a UV hyperchromicity assay. Polycations such as polylysine that interact electrostatically with DNA alter the absorbance and optical rotation characteristics of the nucleic acid [18]. Titration of DNA with unmodified PDL produces a curve with peak UV absorbance corresponding to a 1:1 ratio of lysine monomers to nucleotide residues (Fig. 2). A nearly identical curve is produced when transferrin–polylysine is added to the DNA. The PDL conjugates with ratios of Gd–DTPA per PDL molecule of 10 and 22 show similar effects, suggesting that these modified PDL molecules interact with DNA (Fig. 2). In contrast, PDL conjugates having larger numbers of DTPA chelates (42 and 51) produce no

Table 1. Effect of reagent ratios on modification of poly-D-lysine with DTPA.

DTPA:poly-D-lysine		Unmodified lysine units
Input ratio	Output ratio	per DTPA site
50	10	16.0
100	22	6.7
200	42	3.0
400	51	2.3

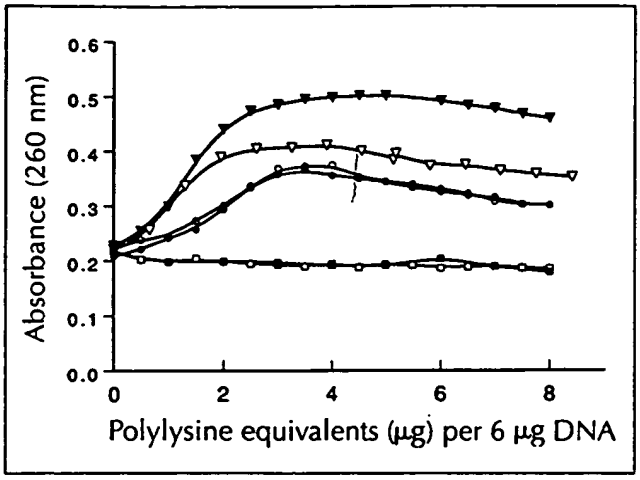

Fig. 2. PDL modified with 10 or 22 Gd chelates per PDL molecule bind to DNA. A UV hyperchromicity assay was used to assess binding of polylysine to DNA. Increasing quantities of PDL or modified polylysine were added to 6 μg plasmid DNA in 1.0 ml HBS while monitoring absorbance at 260 nm. The addition of unmodified PDL (○) or transferrin–polylysine (●) to DNA produces characteristic and nearly identical changes in UV absorbance. Peak absorbance occurs at approximately a 1:1 molar ratio of lysine monomers to nucleotides, corresponding to electro-neutrality and optimal transfection efficiency. Titration of DNA with PDL modified with 10 Gd chelates per PDL molecule (Gd–$DTPA_{(10)}$–PDL; ▽) or 22 Gd chelates (Gd–$DTPA_{(22)}$–PDL; ▼) results in distinct but grossly similar curves. In contrast, Gd–$DTPA_{(42)}$–PDL (□) and Gd–$DTPA_{(51)}$–PDL (■) have minimal effects on DNA absorbance, consistent with a reduced ability of these heavily labeled PDL molecules to interact with DNA.

observable increase in the UV absorbance, suggesting only minimal interaction with the DNA (Fig. 2). Based on these results, the conjugate containing a ratio of 22 Gd–DTPA sites per PDL molecule (Gd–$DTPA_{(22)}$–PDL) was used in subsequent experiments.

Luciferase gene delivery by particles containing DNA, Tf–PL and Gd–DTPA–PDL

Particles were formed by adding varying amounts of transferrin conjugated to poly-L-lysine (Tf–PL) to plasmid DNA encoding the firefly luciferase gene, followed by the addition of a sufficient amount of Gd–$DTPA_{(22)}$–PDL to neutralize the remaining negative charge of the DNA. Tf–PL (3 μg, equivalent to 0.4 μg of unmodified polylysine) was added to 6 μg of DNA to neutralize approximately one tenth of the DNA charge. The remainder of the DNA charge was neutralized by the addition of either 4 μg of unconjugated PDL or an equivalent amount of Gd–$DTPA_{(22)}$–PDL.

High luciferase expression resulted from incubation of the particles containing Gd–DTPA with human erythroleukemic K562 cells in the presence of the lysosomatropic agent, chloroquine (Fig. 3A). It was surprising that the level of gene expression was increased approximately two-fold over that obtained using particles lacking Gd–DTPA–PDL (Fig. 3B). This enhancement of gene expression may be due to neutralization of a portion of the charges on the polylysine backbone by the Gd–DTPA chelates. In the absence of chloroquine, gene expression was not observed (Fig. 3D).

Our data indicate that gene delivery takes place through the transferrin receptor via receptor-mediated endocytosis. Neither unmodified polylysine [8] nor Gd–DTPA–PDL (data not shown) on their own facilitate the transfer of DNA into K562 cells. Furthermore, gene expression is blocked by adding iron-loaded transferrin to the medium during transfection (Fig. 3C) but not by adding apotransferrin, which does not bind to the receptor at neutral pH [19].

Complexes containing DNA, Tf–PL and Gd–DTPA–PDL enhance the MRI contrast of cells

Having demonstrated that DNA complexes containing Tf–PL and Gd–DTPA–PDL are effective gene-delivery vehicles, we then examined the ability of these particles to enhance MRI contrast of transfected K562 cells *in vitro*. Cells treated with these particles were washed extensively in hepes-buffered saline (HBS) and gently loaded into sealed glass capillary tubes, and T_1-weighted images (T_R/T_E = 200 ms/13 ms) were acquired (Fig. 4). MRI contrast enhancement is clearly observed in cells exposed to the Gd-containing particles (Fig. 4a). In contrast, cells exposed to particles lacking Gd–DTPA–PDL show no such enhancement (Fig. 4b). Enhancement of

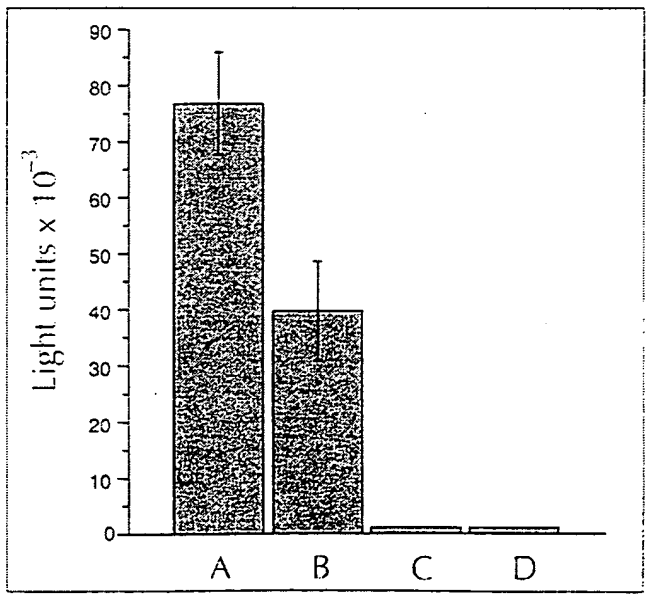

Fig. 3. Transfection of cells is specific and is enhanced using particles containing Gd-modified PDL. Ternary complexes were formed with 6 μg DNA, 3 μg Tf–PL and 4 μg (unmodified lysine equivalents) of either Gd–$DTPA_{(22)}$–PDL (column A) or PDL (column B). Each bar represents the average of values obtained in five independent transfection experiments using K562 cells and normalized to 10^6 cells per experiment. Using Gd–$DTPA_{(22)}$–PDL-containing particles, gene expression was measured in the presence of 20 μg of iron-loaded transferrin to show the effect of competitive uptake (column C) and in the absence of chloroquine to assess the mechanism of cytoplasmic delivery (column D). In these experiments, 1 ng of luciferase produces ~10^5 light units.

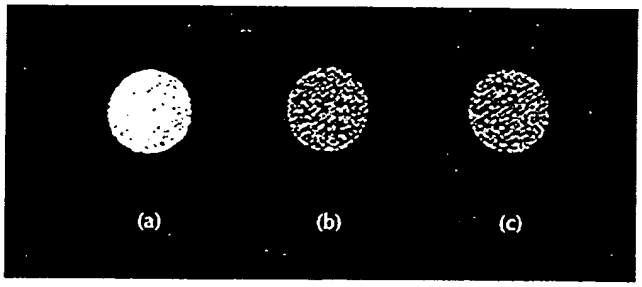

Fig. 4. The ternary complex containing Gd–DTPA–PDL delivers the MRI contrast agent to K562 cells via the transferrin receptor. A two-dimensional horizontal MRI slice is shown through three capillary tubes (2 mm inner diameter) loaded with labeled or control K562 cells. **(a)** Cells exposed to the ternary complex containing 6 μg DNA, 3 μg Tf–PL and 4 μg Gd–$DTPA_{(22)}$–PDL. Bright spots represent aggregates of contrast-agent-containing cells. Mean intensity: 232 ± 25. **(b)** Cells treated with control complexes containing 4 μg unmodified PDL in lieu of the gadolinium-modified PDL. Mean intensity: 121 ± 30. **(c)** Cells treated as in (a) with the addition of 20 μg iron-loaded transferrin to competitively block uptake of the ternary complex. Mean intensity: 126 ± 30. A 10 μg ml^{-1} standard solution of Gd–DTPA in water produces a mean intensity of 142 ± 32, although it should be noted that MRI is extremely sensitive to specific experimental conditions, including solvent composition and viscosity.

the MRI contrast of cells is competitively inhibited by the addition of iron-loaded transferrin, indicating that the observed effect is receptor-specific (Fig. 4c). The magnetic resonance imaging results obtained in these experiments were the same in the presence or absence of chloroquine, suggesting that escape from the lysosome is not necessary for MRI enhancement.

In summary, we have shown that modified polycations can be used to deliver DNA and other molecules to cells at high efficiency. Previous work has shown that ~20 transferrin molecules per complex are necessary to impart receptor-specific uptake of DNA [8]. This number of target molecules can be achieved with as little as 10–15 % of the polylysine necessary to condense a 6 kb plasmid. The remaining 90 % of the DNA negative charge can be neutralized by the addition of approximately 4 μg of unmodified polylysine, or an equivalent amount of modified polylysine. Previous work has demonstrated the utility of modifying this neutralizing polylysine with agents that disrupt endosomes, thus enhancing the escape of the DNA from lysosomal degradation [13–15]. The coupling of adenovirus and influenza virus fusogenic peptides has been especially fruitful in this regard, resulting in high levels of gene expression. In our experiments, the addition of chloroquine during transfection served the purpose of preventing acidification of the endosome and reducing the destruction of the DNA following endocytosis. Thus, most of the polylysine used to neutralize the DNA could be substituted with chelated Gd^{3+}. Inclusion in the complex of another polylysine component that is conjugated to a fusogenic peptide may give high levels of gene expression even in the absence of chloroquine.

Using the Gd–DTPA–PDL which we have synthesized, 22 Gd^{3+} ions are chelated to each PDL molecule, corresponding to ~1200 Gd^{3+} ions per plasmid molecule in the ternary complex. This greatly exceeds the number of contrast agents that can be attached directly to a monoclonal antibody. Moreover, the fast rate of transferrin receptor turnover (2 x 10^4 transferrin molecules internalized per minute) results in the uptake of extremely large numbers of contrast agent molecules in this system [20]. This uptake is enhanced *in vitro* when the particles are at relatively high concentrations and, prior to transfection, cells are starved for iron, inducing an increase in the number of transferrin receptors on the cell surface [8].

Significance

The delivery of genes to specific cells and tissues for therapeutic and research purposes is of increasing importance. A new method to track the delivery of vectors for gene transfection and genetic therapy is imagined based on the work described in this report. This method combines a magnetic resonance imaging (MRI) contrast agent and a receptor-targeted gene delivery vehicle.

MRI is an ideal tool for non-invasive monitoring of human and experimental subjects. We have synthesized a new class of MRI contrast agents capable of transfecting genes into cells and enhancing the MRI contrast of these targeted cells *in vitro*. The particles are composed of DNA, polylysine attached to transferrin, and polylysine modified with a paramagnetic contrast agent. Modification of this strategy should produce particles capable of functioning *in vivo*. Such particles could be composed of i) DNA encoding a reporter or therapeutic gene, ii) polylysine modified with transferrin or with an alternative cell-targeting molecule, such as a monoclonal antibody, iii) polylysine modified with fusogenic peptides to facilitate release from the endosomal pathway and iv) polylysine (D or L forms) modified with Gd chelates. Co-transport of DNA and MRI contrast agents of this type should result in high levels of gene expression. These particles may prove extremely useful in targeting specific cells *in vivo* as they offer a means other than marker gene expression to monitor construct uptake. With such a tool, clinicians and experimentalists may be able to non-invasively monitor delivery of genetic therapeutic agents in real time.

Materials and methods

Preparation of Tf–PL

Human transferrin (Tf, Sigma) was conjugated to poly-L-lysine (PL) with an average chain length of 220 residues (Sigma) via sialic acid residues on transferrin as described [21], or the Tf–PL conjugate was purchased directly from Sigma. Modified polylysine (Tf–PL) with average molar ratios of transferrin to

polylysine of 2.2–2.5 was dissolved in HBS (20 mm Hepes, 150 mM NaCl, pH 7.4) at a working concentration of 100 μg ml^{-1} for use in subsequent experiments.

Preparation of DTPA–PDL

PDL of average chain length 180 was purified by FPLC size-exclusion chromatography employing a 10 x 30 Pharmacia Superdex 75 column using 0.15 M NaCl, 0.15 M NaPi, pH 7.0 buffer. The purified PDL was desalted by gel filtration using Sephadex G-25 and dried *in vacuo*. The dry PDL was weighed and the total number of lysine monomer units verified spectrophotometrically via a ninhydrin assay [22]. PDL (0.01 M) was placed in a round-bottomed flask and dissolved in 0.05 M HEPES, pH 9.5. Freshly prepared DTPA anhydride was added slowly with stirring. The pH of the solution was monitored and maintained at 9.5 throughout the course of the reaction using a 3 % NaOH solution. The molar ratios of DTPA-anhydride to PDL used in these experiments was 50:1, 100:1, 200:1 and 400:1. The reaction was allowed to proceed at room temperature for 1 h after complete addition of the anhydride, and the crude material was purified by FPLC.

Determination of DTPA content by fluorescence titration

The binding of lanthanide metals such as gadolinium, europium and terbium to chelating agents produces fluorescence with large Stoke's shifts and fluorescence lifetimes. Eu^{3+} (unlike Gd^{3+}) rapidly coordinates with DTPA derivatives, producing a strong fluorescent signal that was monitored to assay DTPA content of the various DTPA–PDL conjugates. Samples of the purified DTPA–PDL were diluted in 0.05 M HEPES, pH 7.4 to a final concentration of 2–4 x 10^{-5} M. Small volumes (<20 μl) of varying molarities of $EuCl_3$ were added to the 1-ml samples to titrate the free DTPA ligand with minimal dilution of the sample. After incubation for 2 min at room temperature, each sample was transferred to a Hitachi Model 2400 spectrofluorimeter. Measurements were made using excitation at 394 nm and monitoring emission at 593 and 616 nm. Molar ratios of DTPA per PDL were calculated using the fluorescence titration data and the polylysine quantification that was based on ninhydrin as an assay for unmodified lysine side-chains [22].

Preparation of Gd–DTPA–PDL

Based on the chelator content determined by Eu titration, a 1.1 molar excess of $GdCl_3$ was added to 1 mg ml^{-1} aliquots of the DTPA–PDL in 0.05 M citrate buffer, pH 6.0. The chelation proceeded for 5 h at 70 °C, and was followed by desalting chromatography with Sephadex G25 to remove unbound Gd. Gd–DTPA–PDL samples were dissolved in HBS at a working concentration of 100 μg ml^{-1}.

Preparation of MRI/gene delivery particles

A 6 kilobase-pair plasmid containing the *Photinus pyralis* luciferase gene under control of the SV40 T-antigen enhancer was purchased from Promega (GeneLight control plasmid). This plasmid was grown in *Escherichia coli*, and DNA was prepared in 100 μg quantities using a commercially available procedure (Qiagen, Midi-100). The supercoiled DNA plasmids were dissolved at a concentration of 100 μg ml^{-1} in 10 mM Tris, 1 mM ethylenediaminetetraacetic acid, pH 7.4 (TE). Plasmid DNA (6 μg) in 430 μl of HBS was mixed with 3 μg of Tf–PL in 30 μl HBS. After 5 min at room temperature, various amounts (generally 4 μg equivalents of polylysine) of Gd–DTPA–PDL with differing degrees of Gd substitution were added to the sample which was brought to 500 μl with HBS and allowed to incubate at room temperature for 30 min.

Transfections

K562 human leukemia cells (ATCC) were grown in suspension in RPMI 1640 medium (Gibco) containing 10 % fetal calf serum (Hyclone) containing 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM glutamine, to a maximum density of 5 x 10^5 cells ml^{-1}. Eighteen to twenty-four hours prior to transfections, cells were placed in fresh medium containing 50 μM deferroxamine (Sigma) to increase surface presentation of transferrin receptors as described [12]. Approximately ten minutes prior to transfection, the cells were washed with fresh medium containing deferroxamine and placed in a 12-well dish at 250 000 cells per ml, 2 ml per well. Chloroquine (10 mM) in H_2O was added to a final concentration of 100 μm.

The ternary particle in HBS was added slowly to the K562 cells, which were incubated at 37 °C for 18–24 h. In some cases, iron-loaded transferrin (Sigma) was added with the ternary complex to competitively inhibit specific uptake of the particles. The cells were then washed twice with fresh medium without chloroquine or deferroxamine and resuspended at 250 000 cells ml^{-1} in fresh medium. After 18 h, the cells were washed twice in HBS and either gently loaded into a glass capillary tube for MR imaging or assayed for luciferase expression. Gene expression was monitored by lysing cells in 100 μl detergent lysis buffer (Clontech). Cell lysate (20 μl) was assayed in a 1 ml standard cell using a luminometer and luciferin substrate from Analytical Luminescence Laboratory. These results were normalized per 10^6 cells and confirmed by parallel measurements using a scintillation counter (Beckman Instruments).

MRI image acquisition

MRI images were acquired using an 11.7 Tesla Bruker AMX 500 MHz NMR spectrometer with microimaging accessory. Glass capillary tubes (2 mm inner diameter) containing ~10^6 cells per tube were immobilized in a plexiglass rack and loaded into the MRI bore. Images were acquired using a multi-slice 3D spin echo protocol where T_R = 200 ms, T_E = 13 ms and the pulse width was set at 9.6 μs. This protocol results in a T_1-weighted image and a 512 by 512 by 32 data array.

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References

1. Tootell, R.B.H., *et. al.*, & Belliveau, J.W. (1995). Functional analysis of human MT and related visual cortical areas using magnetic resonance imaging. *J. Neurosci.* **15**, 3215–3230.
2. Jacobs, R.E. & Fraser, S.E. (1994). Magnetic-resonance microscopy of embryonic-cell lineages and movements. *Science* **263**, 681–684.
3. Alexander, V. (1995). Design and synthesis of macrocyclic ligands and their complexes of lanthanides and actinides. *Chem. Rev.* **95**, 273–342.
4. Lauffer, R.B. (1991). Targeted relaxation enhancement agents for MRI. *Magn. Reson. Med.* **22**, 339–342.
5. Bogdanov, A.A., Jr, Martin, C., Weissleder, R. & Brady, T.J. (1994). Trapping of dextran-coated colloids in liposomes by transient binding to aminophospholipid preparation of ferrosomes. *Biochim. Biophys. Acta* **1193**, 212–218.
6. Shen, T., Weissleder, R., Papisov, M., Bogdanov, A.A., Jr & Brady, T.J. (1993). Monocrystalline iron-oxide nanocompounds (mion) physicochemical properties. *Magn. Reson. Med.* **29**, 599–604.
7. Wiener, E.C., *et. al.*, & Lauterbur, P.C. (1994). Dendrimer-based metal chelates. A new class of magnetic-resonance-imaging contrast agents. *Magn. Reson. Med.* **31**, 1–8.
8. Cotten, M., Wagner, E. & Birnstiel, M.L. (1993). Receptor-mediated

transport of DNA into eukaryotic cells. *Methods Enzymol.* **217**, 618–644.
9. Perales, J.C., Ferkol, T., Beegen, H., Ratnoff, O.D. & Hanson, R.W. (1994). Gene transfer *in vivo*: sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake. *Proc. Natl. Acad. Sci. USA* **91**, 4086–4090.
10. Chowdhury, N.R., Wu, C.H., Wu., G.Y., Yerneni, P.C., Bommineni, V.R. & Chowdhury, J.R. (1993). Fate of DNA targeted to the liver by asialoglycoprotein receptor-mediated endocytosis *in vivo*. Prolonged persistence in cytoplasmic vesicles after partial hepatectomy. *J. Biol. Chem.* **268**, 11265–11271.
11. Cristiano, R.J., Smith, L.C. & Woo, S.L.C. (1993). Hepatic gene therapy. Adenovirus enhancement of receptor-mediated gene delivery and expression in primary hepatocytes. *Proc. Natl. Acad. Sci. USA* **90**, 2122–2126.
12. Wagner, E., Cotten, M., Foisner, R. & Birnstiel, M.L. (1991). Transferrin polycation DNA complexes — the effect of polycations on the structure of the complex and DNA delivery to cells. *Proc. Natl. Acad. Sci. USA* **88**, 4255–4259.
13. Michael, S.I., Huang, C.H., Rømer, M.U., Wagner, E., Hu, P.C. & Curiel, D.T. (1993). Binding-incompetent adenovirus facilitates molecular conjugate-mediated gene-transfer by the receptor-mediated endocytosis pathway. *J. Biol. Chem.* **268**, 6866–6869.
14. Plank, C., Oberhauser, B., Mechtler, K., Koch, C. & Wagner, E. (1994). The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene-transfer systems. *J. Biol. Chem.* **269**, 12918–12924.
15. Plank, C., Zatloukal, K., Cotten, M., Mechtler, K. & Wagner, E. (1992). Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artificial tetra-antennary galactose ligand. *Bioconjugate Chem.* **3**, 533–539.
16. Brandt, K.D., Schnobrich, K.E. & Johnson, D.K. (1991). Characterization of antibody–chelator conjugates: determination of chelator content by terbium fluorescence titration. *Bioconjugate Chem.* **2**, 67–70.
17. Shapiro, J.T., Leng, M. & Felsenfeld, G. (1969). Deoxyribonucleic acid–polylysine complexes. Structure and nucleotide specificity. *Biochemistry* **8**, 3219–3232.
18. Minagawa, K., Matsuzawa, Y., Matsumoto, M. & Doi, M. (1991). Direct observation of the biphasic conformational change of DNA induced by cationic polymers. *FEBS Lett.* **295**, 67–69.
19. Zenke, M., Steinlein, P., Wagner, E. Cotten, M., Beug, H. & Birnstiel, M.L. (1990). Receptor-mediated endocytosis of transferrin polycation conjugates: an efficient way to introduce DNA into hematopoietic cells. *Proc. Natl. Acad. Sci. USA* **87**, 3655–3659.
20. Huebers, H.A. & Finch, C.A. (1987). The physiology of transferrin and transferrin receptors. *Physiol. Rev.* **67**, 520–582.
21. Wagner, E., Cotten, M., Mechtler, K., Kirlappos, H. & Birnstiel, M.L. (1991). DNA-binding transferrin conjugates as functional gene-delivery agents. Synthesis by linkage of polylysine or ethidium homodimer to the transferrin carbohydrate moiety. *Bioconjugate Chem.* **2**, 226–231.
22. Sarin, V.K., Kent, S.B.H., Tam, J.P. & Merrifeld, R.B. (1981). Quantative monitoring of solid-phase synthesis by the ninhydrin reaction. *Anal. Biochem.* **117**, 147–157.

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Expedited Articles

A Modular Lymphographic Magnetic Resonance Imaging Contrast Agent: Contrast Enhancement with DNA Transfection Potential

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A gadolinium-chelated liposomal contrast agent has been prepared, and magnetic resonance imaging (MRI) efficacy has been examined by indirect magnetic resonance lymphography. A lipidic *N,N'*-dimethylethylenediamine derivative (**4**) containing a 10,12-diyne-diacyl domain was treated with DTPA anhydride followed by $GdCl_3$ complexation. The complex was confirmed using MALDI spectrometry. An equimolar mixture of the Gd–chelate lipid and a commercially available diyne-PE was formulated as a liposome suspension and irradiated with UV light prior to imaging experiments. Subcutaneous injection of the liposomal gadolinium agent and subsequent MRI of rabbit axillary and popliteal lymph nodes revealed significant contrast enhancement up to 4 h postinjection. To explore the possibility of imaging a DNA transfection event, the gadolinium contrast mixture was formulated with the cationic transfection lipid DOTAP and complexed with the reporter gene encoding luciferase. DNA transfection studies on the NIH3T3 cell line confirmed the transfection activity of the dual-purpose contrast agent and exemplified the potential toward development of an imaging and DNA delivery vehicle.

Since the advent of magnetic resonance imaging (MRI) for medical diagnostics, there has been continued interest in the design and synthesis of paramagnetic complexes for contrast enhancement.[1] Among the MRI contrast agents that have been developed, gadolinium(III) complexes have been shown to be highly effective in their imaging properties.[2] However, the clinical utility of a contrast agent depends not only on absolute contrast-enhancing properties but also on the pharmacokinetics that determine distribution and concentration of the contrast agent in target tissues. Indirect MR lymphography, a technique in which subcutaneously injected contrast material selectively accumulates within regional lymph nodes, has recently gained attention as an approach for diagnosing and staging cancer.[3] The most widely available gadolinium contrast agent, gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA), is not used for lymphographic MRI because of its rapid distribution within the extracellular space and rapid renal clearance.[4] To overcome this limitation, one approach has been to incorporate the gadolinium into liposomal lamellae.[5,6] Incorporation of the paramagnetic species into liposomes can result in increased accumulation within the lymph nodes by intranodal macrophage uptake of particulate species. We seek to extend this approach by introducing a polymerizable lipid formulation that is capable of binding gadolinium as well as binding a polynucleotide.[7] Lipid-mediated DNA delivery as a means for gene therapy is a developing modality for the treatment of cancer and other diseases,[8] and the combination of this methodology with selective, contrast-enhanced MRI would improve its utility. Visualization of polynucleotide localization within lymph nodes by gadolinium contrast enhancement would be a rapid, noninvasive means of monitoring treatment, were the same agent also capable of simultaneously serving as the gene transfection agent. A report by Meade et al. on the cotransport of DNA and DTPA-Gd using polylysine in cell culture experimentation supports the feasibility of this approach.[9]

We report herein the synthesis of a prototypic gadolinium-chelated lipid and its modular incorporation with a commercially available DNA-binding lipid to yield a dual-purpose liposome formulation for lymphographic imaging and treatment. Preliminary imaging and DNA transfection properties are presented.

Results and Discussion

Synthesis. The synthesis of the gadolinium chelate lipid is shown in Scheme 1. Treatment of 10,12-octadecadiynoyl chloride (**2**) with 3-bromo-1,2-propanediol yields bisester **3**. The bromide of **3** is readily displaced by treatment with excess *N,N'*-dimethylethylenediamine at elevated temperature. Reaction of **3** using fewer equivalents of diamine or longer reaction times affords significantly lower yields of diamine **4**. Amide formation by reaction of **4** with excess DTPA-anhydride in pyridine is followed by hydrolysis of unreacted anhydride. The corresponding amide mixture[10] was reacted with Gd(III) according to the procedure by Bednarski et al.,[7a] and the resultant chelate **5** was analyzed by matrix-assisted laser desorption ionization (MALDI) spectrometry. The MALDI spectrum of **5** confirms an isotopic distribution characteristic of a gadolinium species, centered around the expected molecular weights.[11]

Magnetic Resonance Imaging. To determine the contrast properties of the gadolinium-bound lipid **5**,

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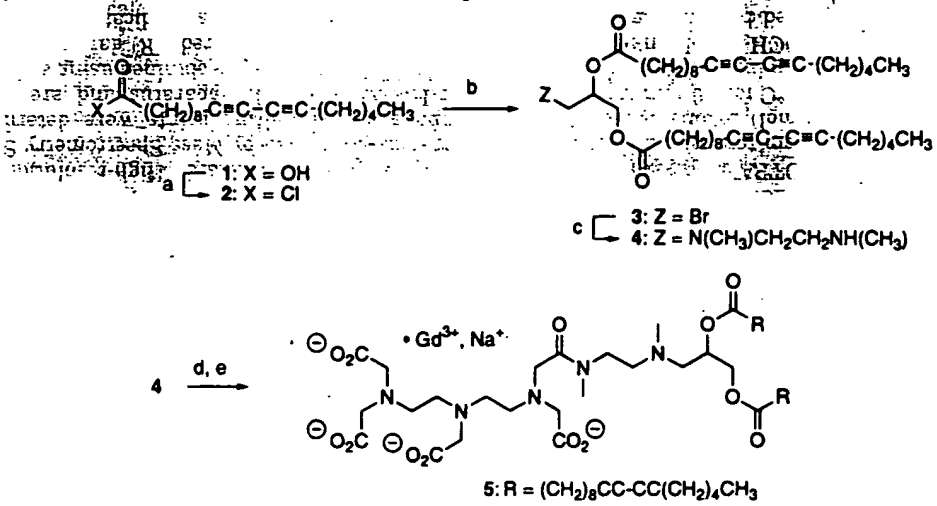
EXHIBIT B

Scheme 1. Synthesis of Polymerizable Gd–Chelate Lipid[a]



[a] Reagents and conditions: (a) $(CO)_2Cl_2$, $CHCl_3$, reflux; (b) $BrCH_2CH(OH)CH_2OH$ (0.45 equiv), Et_3N (2.2 equiv), cat. DMAP, CH_2Cl_2; (c) $HN(CH_3)CH_2CH_2NH(CH_3)$ (15 equiv), DMF, 75 °C, 45 min; (d) i. DTPA-anhydride (3.5 equiv), pyridine, 50 °C, 12 h, ii. H_2O; (e) $GdCl_3 \cdot 6H_2O$, $NaOCH_3$, CH_3OH, 60 °C, 30 min.

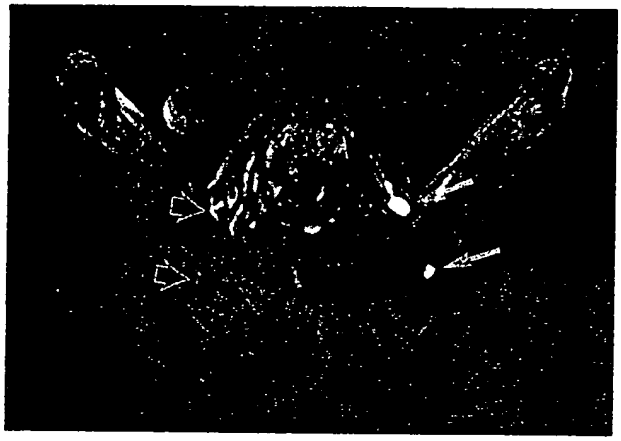


Figure 1. T_1-weighted transaxial MR image of contrast-enhanced axillary and superficial cervical lymph nodes in a normal New Zealand White rabbit (solid arrows). This image was acquired approximately 120 min after subcutaneous injection of 0.4 mL of the Gd-bound suspension (17×10^{-6} mol of Gd) into the ipsilateral metacarpus. The unenhanced contralateral nodes are poorly visualized (open arrows).

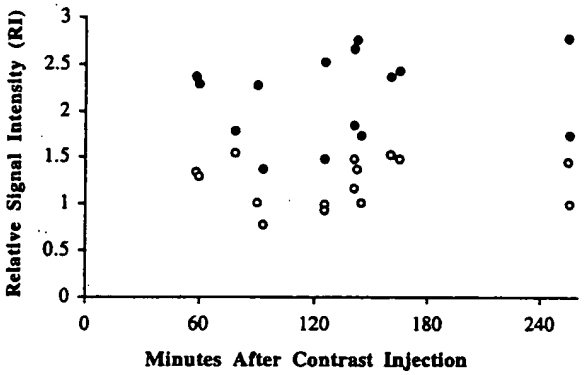


Figure 2. Relative signal intensity (RI) of lymph nodes on T_1-weighted spin–echo magnetic resonance images versus time postcontrast injection: (●) RI of all contrast-enhanced lymph nodes at each time point analyzed, (○) RI of corresponding unenhanced contralateral lymph nodes at each time point.

preliminary MRI studies were performed on three normal rabbits. The contrast material, polymerized liposome formulations containing equimolar quantities of lipid **5** and 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphoethanolamine (**6**),[12] serving as a polymer matrix lipid, was prepared by sonication of the aqueous lipid suspension followed by UV photolysis (0 °C, 1 h). Each animal was anesthetized and then injected subcutaneously with an empirically determined dose of contrast material at the level of the left metacarpus or metatarsus to enhance the axillary/cervical or popliteal lymph nodes, respectively. Earliest images were acquired beginning approximately 1 h after contrast administration and at 30–90-min intervals for up to 260 min postinjection. A representative image is presented in Figure 1.

Images were obtained from at least two time points for each lymph node. Using the current formulation to assess seven lymph nodes in three rabbits, relative signal intensity (RI, mean signal intensity of the node divided by the mean signal intensity of surrounding muscle tissue) increased from 1.16 in unenhanced nodes to 2.19 in contrast-enhanced nodes at peak enhancement ($p > 0.0002$, two-sample t-test) (Figure 2). This resulted in a 91.7% peak percent contrast enhancement, PCE. Peak enhancement generally occurred 60–130 min after contrast injection, and enhancement persisted up to 4 h postinjection. This result demonstrates the usefulness of the polymerized liposome construct in promoting lymph node retention of gadolinium relative to Gd-DTPA preparations.

DNA Transfection Using the Gd–Liposome Formulation. Cationic lipids have been demonstrated to facilitate intracellular uptake of DNA in a number of in vitro and in vivo studies.[13,14] Typically, a cationic liposome suspension is prepared and treated with DNA so that the resultant lipid–DNA complex (referred to as a lipoplex[15]) retains an overall positive charge. Direct application of the lipoplex to cultured cells then can result in intracellular delivery and expression of the DNA. To see the influence of gadolinium incorporation on DNA transfection, a luciferase transfection assay was conducted using the methods we have previously reported.[16] Liposomes formulated using *N*,*N*,*N*-trimethyl-*N*-(1-(2,3-dioleoyl)propyl)ammonium iodide (DOTAP[17])

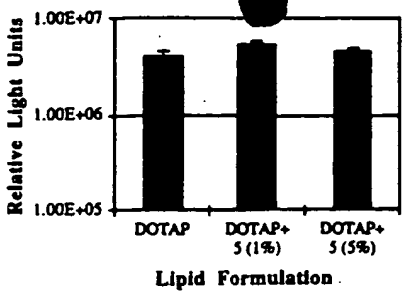


Figure 3. Comparison of lipid-mediated DNA transfection using a 2:1 molar charge ratio (DOTAP charge to DNA phosphate charge) to transfect NIH3T3 cells. DNA transfections were performed in quadruplicate, and the results are summarized in bar graph form as the mean (n = 4) and standard deviation of total luciferase light units obtained as described in the Experimental Section.

and gadolinium-chelate lipid **5** were used to bind pND-CLux, plasmid DNA encoding the firefly luciferase gene.[18] The resultant lipoplexes were used to transfect NIH3T3 murine fibroblast cells (Figure 3) at an optimal DOTAP:DNA molar charge ratio. Liposomes formulated with chelate **5** showed activity comparable to the control experiment of DOTAP. The presence of up to 5% gadolinium did not reduce the relative activity of the DOTAP lipoplex. Our results support the development of a dual-purpose contrast agent in that the selected MRI agent does not interfere with in vitro transfection. Studies are underway to extend the application to in vivo transfection of rabbit lymph nodes.

In conclusion, we have shown the possibility for development of a dual-purpose imaging and DNA delivery vehicle. The inherent modular construction of a lipoplex formulation was exploited by incorporation of a gadolinium–chelate lipid and a DNA-binding lipid. The resultant particle was shown to facilitate DNA transfection. Furthermore, a liposomal formulation of the gadolinium lipid was shown to have excellent in vivo contrast enhancement with prolonged lymph node retention.

Experimental Section

General. CH_2Cl_2 was distilled from calcium hydride immediately prior to use. MeOH was heated over Mg turnings for 12 h, and distilled prior to use. All amine reagents were distilled from CaH_2. All reactions were carried out under an atmosphere of argon. The acyl halides were prepared using a modification of the experimental reported by Clark et al.[19] Column chromatography was carried out using 230–400 mesh silica gel, slurry packed in glass columns, eluting with the solvents indicated. Yields were calculated for material judged to be homogeneous by TLC and NMR. TLC was performed on Merck Kieselgel 60 F_{254} plates, staining with a solution of phosphomolybdic acid in ethanol containing 3% concentrated H_2SO_4.

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) were recorded using a General Electric QE-300 spectrometer with residual undeuterated solvent (δ 7.26 for $CHCl_3$, δ 4.78 for CD_3OH) serving as the internal standard. High-resolution mass spectrometry was performed by Mass Spectrometry Service Lab, Minneapolis, MN. Melting points were determined on a Thomas-Hoover Uni-melt apparatus and are uncorrected. Ultraviolet (UV) spectra were obtained using a HP8450A UV/vis spectrophotometer. Infrared (IR) data were obtained on neat samples unless otherwise indicated using a Mattson Galaxy Series FTIR 3000 instrument.

3-Bromo-1,2-bis(10,12-octadecadiynoyl)propane (3). Oxalyl chloride (6.32 mL, 72.5 mmol) was added slowly to a solution of 10,12-octadecadiynoic acid (**1**) (10.0 g, 36.3 mmol) in chloroform (13.5 mL). The resultant reaction solution was stirred for 10 min at room temperature and then heated at reflux temperature for 20 min. All solvents were then removed by distillation to afford crude acyl chloride (**2**) as a brown oil. The crude material was dissolved in CH_2Cl_2 (5 mL) and transferred via cannula to a solution of 3-bromo-1,2-propanediol (1.32 mL, 15.1 mmol), Et_3N (4.54 mL, 32.6 mmol), and DMAP (0.181 g, 1.48 mmol) in CH_2Cl_2 (10 mL) at 0 °C. After 10 min, the reaction mixture was warmed to room temperature and stirred overnight. The reaction mixture was then diluted with CH_2Cl_2, washed with saturated $NaHCO_3$, H_2O, and saturated brine, and dried (Na_2SO_4). The solvents were removed by rotary evaporation, and the residue was chromatographed using silica gel (hexane–ethyl acetate, 9:1) to give 9.71 g (96%) of **3** as an oil: IR 2930, 2855, 2253, 2145, 1742 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.17 (m, 1H), 4.31 (dd, J = 11.8, 4.3 Hz, 1H), 4.18 (dd, J = 11.8, 5.6 Hz, 1H), 3.46 (m, 2H), 2.28 (m, 4H), 2.19 (m, 8H), 1.57 (m, 4H), 1.45 (m, 8H), 1.26 (m, 24H), 0.85 (t, J = 7.35 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 172.6, 172.2, 77.1, 77.0, 69.9, 65.6, 65.5, 62.9, 33.8, 31.0, 30.0, 29.3, 29.2, 29.1, 29.0, 28.9, 28.7, 28.6, 28.4, 28.3, 28.1, 24.8, 23.7, 22.2, 19.1, 18.3, 13.9; HRMS calcd for $C_{39}H_{59}BrO_4$ [M^{79}Br (49.77) + NH_4]$^+$ 688.3941, found 688.4003; calcd for $C_{39}H_{59}BrO_4$ [M^{81}Br (41.40) + NH_4]$^+$ 690.3920, found 690.3935.

3-(*N*-Methyl-*N*-(2-(*N*'-methylamino)ethyl)amino)-1,2-bis(10,12-octadecadiynoyl)propane (4). *N,N*'-Dimethylethylenediamine (3.57 mL, 33.5 mmol) was added to a solution of **3** (1.50 g, 2.24 mmol) in DMF (11.2 mL) at room temperature. The reaction mixture was heated to 75 °C and stirred for 45 min. The DMF was removed by distillation, and the residue was diluted with CH_2Cl_2, washed with saturated $NaHCO_3$, H_2O, and saturated brine, and dried (Na_2SO_4). The solvent was removed by rotary evaporation, and the residue was chromatographed using silica gel (CH_2Cl_2–MeOH, gradient of 99:1 to 95:5) to give 0.739 g (49%) of **4** as an oil: IR 2931, 2856, 2256, 2158, 1738 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 5.10 (m, 1H), 4.34 (dd, J = 12.0, 3.1 Hz, 1H), 4.07 (dd, J = 12.0, 6.2 Hz, 1H), 2.95 (m, 2H), 2.78 (m, 2H), 2.74 (s, 3H), 2.53 (d, J = 6.5 Hz, 2H), 2.34 (s, 3H), 2.29 (m, 4H), 2.23 (m, 8H), 1.59 (m, 4H), 1.51 (m, 8H), 1.29 (m, 24H), 0.88 (t, J = 7.1 Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 173.5, 173.4, 77.0, 76.5, 69.5, 65.2, 65.1, 63.5, 57.8, 53.6, 46.6, 42.8, 34.2, 34.0, 33.2, 30.8, 29.0, 28.9, 28.8, 28.6, 28.5, 28.3, 28.2, 27.9, 24.7, 22.0, 19.0, 13.8; HRMS calcd for $C_{43}H_{70}N_2O_4$ [M + H]$^+$ 679.5413, found 679.5398.

3-(*N*-Methyl-*N*-(2-(*N*'-DTPA-amido-*N*'-methylamino)-ethyl)amino)-1,2-bis(10,12-octadecadiynoyl)propane and Gadolinium Complexation (5). DTPA-anhydride (2.34 g, 6.54 mmol) was added to a solution of **4** (1.27 g, 1.87 mmol) in pyridine (9.35 mL) at room temperature. The reaction mixture was heated to 50 °C and stirred overnight. The cooled reaction mixture was filtered to remove unreacted DTPA-anhydride, and distilled water (5 mL) was added to the filtrate. The solvents were removed by distillation under reduced pressure. The crude product was dissolved in DMSO. Any undissolved solids were removed by filtration. The DMSO was then removed in vacuo to afford 1.77 g (90%) of the DTPA conjugate as a tan solid: mp 183–184 °C; IR 3414, 2931, 2864, 2256, 2160, 1742, 1641 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 5.13 (m, 1H), 4.27 (m, 1H), 4.02 (m, 1H), 3.76 (m, 2H), 3.63 (m, 2H), 3.47 (s, 8H), 2.92 (s, 3H), 2.51 (s, 3H), 2.32 (m, 4H), 2.27 (m, 8H), 1.45 (m, 12H), 1.30 (m, 24H), 0.86 (t, J = 6.8 Hz, 6H); ^{13}C NMR (DMSO-d_6) δ 172.8, 172.7, 172.6, 76.9, 69.5, 63.5, 57.8, 54.1, 46.9, 42.7, 34.2, 34.0, 33.5, 31.7, 29.6, 29.4, 29.3, 29.1, 29.0, 27.1, 27.1, 27.0, 24.8, 22.5, 13.9; HRMS calcd for $C_{57}H_{91}N_5O_{13}$ [M + Na]$^+$ 1076.6511, found 1076.6517.

To a suspension of the DTPA conjugate (1.00 g, 0.950 mmol) in MeOH (2.38 mL) at room temperature was added $GdCl_3 \cdot 6H_2O$ (0.353 g, 0.950 mmol) in one portion. The reaction mixture was heated to 60 °C for 30 min, and the solution pH was maintained near 7.0 by immediate and continuous monitoring and addition of $NaOCH_3$ (1.6 M in methanol) as needed. The methanol was removed by rotary evaporation, and the residue was redissolved in anhydrous methanol (ca. 5 mL). Undissolved solids were removed by filtration, and the filtrate was concentrated to afford 1.18 g of **5** as a tan solid: mp 258

°C dec; IR 3400, 2928, 2856, 2253, 2156, 1739, 1735, 1609 cm^{-1}; UV 224, 238, 254 nm; HRMS calcd for $C_{57}H_{87}GdN_5O_{13}$ 1253.5337, found 1253.5306.

Preparation of Liposomes for MRI. Sterile water (1.3 mL) was added to a vial containing **5** (51.4 mg, 0.0425 mmol) and an equimolar amount of the commercially available 1,2-bis(10,12-tricosadiynoyl)-*sn-glycero*-3-phosphoethanolamine. The suspension was vortex-mixed for 2 min and sonicated for 45 min (Laboratory Supplies Co., Inc., model G112SP1T). The liposome formulation was then transferred to a Petri dish using sterilized water (0.1 mL) and photolyzed (Hanovia Hg lamp; 254, 313, 366 nm) for 65 min at 0 °C (ice). The photolyzed preparation was stored overnight at −4 °C prior to the MRI.

Preparation of Liposomes for DNA Transfection. A chloroform solution of DOTAP (1 μmol) and a methanol solution of gadolinium complex **5** (0.01 or 0.05 μmol) were combined in a 3.7-mL sample vial. The solvents were removed via rotary vacuum evaporation, and the resulting thin lipid film was placed under vacuum overnight. Sterile water (1 mL) was added, and the thin lipid film was hydrated by briefly warming at 60 °C under argon with subsequent vortex mixing. The resultant lipid suspension was used for DNA transfection within 3 h of hydration.

Magnetic Resonance Imaging. MRI experiments were performed with a quadrature coil on a 1.5-T magnet (Signa 1.5, GE Medical Systems, Milwaukee, WI). Each rabbit was induced and maintained under anesthesia using intramuscularly injected ketamine and xylazine for imaging procedures. T_1-weighted images were obtained 60−240 min after SQ injection of the liposome suspension (0.4 mL, 17 × 10^{-3} mmol of Gd) into the ipsilateral metatarsus and metacarpus. Studies were performed using the following pulse sequence: T_1-weighted spin−echo (SE) TE = 11 ms/Fr, TR = 550 ms, rectangular FOV measuring 22 × 16 cm, 3-mm slice thickness with a 1.5-interslice gap, 512 × 224 matrix size, 3 excitations with chemical shift fat suppression.

DNA Transfection. NIH3T3 cells were obtained from ATCC (CRL 1658), cultured in Dulbecco's Modified Eagle's Medium with 10% calf serum and plated on standard 24-well tissue culture plates 12−24 h prior to transfection. Cells were approximately 80% confluent at the time of transfection. The growth medium was removed by aspiration, and the cells were washed once with 0.5 mL of PBS/well. The liposome−DNA complexes were formed through sequential addition of appropriate amounts of DMEM (serum-free), pNDCLux DNA, and the liposome formulation into a 2 mL Eppendorf tube to a total volume of 800 μL. Typically, 24 μL of a lipid emulsion (1 mM DOTAP) was used to complex 4 μg of DNA to yield a 2:1 DOTAP:DNA phosphate molar charge ratio. A 200-μL aliquot of the resultant transfection complex was added to each well (1 μL DNA/well, 4 wells/sample) and the cells were incubated for 4 h at 37 °C. At this time, 500 μL of growth medium and 10% calf serum was added per well, and the cells were cultured for approximately 48 h prior to lysis and analysis.

Relative luciferase activity was determined by using the Enhanced Luciferase Assay Kit and a Monolight 2010 luminometer (both from Analytical Luminescence Laboratories, Ann Arbor, MI). This was accomplished by directly applying 233.3 μL of concentrated luciferase lysis buffer (final concentration 0.1 M potassium phosphate, pH 7.8, 1% Triton X-100, 1 mM DTT, 2 mM EDTA) to each well and placing the cells on ice for 15 min. Luciferase light emissions from 31 μL of the lysate were measured over a 10-s period, and results are expressed as a function of an assumed total lysate volume of 933.3 μL. Activity is measured as relative light units, which are a function of assay conditions, luciferase concentration, luminometer photomultiplier tube sensitivity, and background. Under the conditions described above, relative light units are related to luciferase protein mass by the equation fg of luciferase = (RLU/48.6) − 824.

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Supporting Information Available: Analytical data and procedures for compounds **3**−**5** (2 pages). Ordering information is given on any current masthead page.

References

(1) Lauffer, R. B. MRI Contrast Agents: Basic Principles. In *Clinical Magnetic Resonance Imaging*, 2nd ed.; Edelman, R. R., Zlatkin, M. B., Hesselink, J. R., Eds.; W. B. Saunders Co.: Philadelphia, 1996; Vol. 1, pp 177−191.

(2) Lauffer, R. B. Paramagnetic Metal Complexes as Water Proton Relaxation Agents for NMR Imaging: Theory and Design. *Chem. Rev. (Washington, D.C.)* **1987**, *87*, 901−927.

(3) (a) Harika, L.; Weissleder, R.; Poss, K.; Zimmer, C.; Papisov, M. I.; Brady, T. J. MR Lymphography with a Lymphotropic T1-Type MR Contrast Agent: Gd-DTPA-PGM. *Magn. Reson. Med.* **1995**, *33*, 88−92. (b) Taupitz, M.; Wagner, S.; Hamm, B.; Dienemann, D.; Lawaczeck, R.; Wolf, K. J. MR Lymphography Using Iron Oxide Particles. Detection of Lymph Node Metastases in the VX2 Rabbit Tumor Model. *Acta Radiol.* **1993**, *34*, 10−15.

(4) (a) Goldstein, E. J.; Burnett, K. R.; Hansell, J. R.; Casaia, J.; Dizon, J.; Farrar, B.; Gelblum, D.; Wolf, G. L. Gadolinium DTPA (An NMR Proton Imaging Contrast Agent): Chemical Structure, Paramagnetic Properties and Pharmacokinetics. *Phys. Chem. Phys. Med. NMR* **1984**, *16*, 97−104. (b) Weinmann, H.-J.; Brasch, R. C.; Press, W.-R.; Wesbey, G. E. Characteristics of Gadolinium-DTPA Complex. *J. Am. Radiol.* **1984**, *142*, 619−624. (c) Brasch, R. C.; Weinmann, H.-J.; Wesbey, G. E. Contrast-Enhanced NMR Imaging: Animal Studies Using Gadolinium-DTPA Complex. *J. Am. Radiol.* **1984**, *142*, 625−630.

(5) (a) Trubetskoy, V. S.; Cannillo, J. A.; Milshtein, A.; Wolf, G. L.; Torchilin, V. P. Controlled Delivery of Gd-Containing Liposomes to Lymph Nodes: Surface Modification May Enhance MRI Contrast Properties. *Magn. Reson. Imaging* **1995**, *13*, 31−37. (b) Trubetskoy, V. S.; Torchilin, V. P. New Approaches in the Chemical Design of Gd-Containing Liposomes for Use in Magnetic Resonance Imaging of Lymph Nodes. *J. Liposome Res.* **1994**, *4*, 961−980. (c) Unger, E.; Fritz, T.; Wu, G.; Shen, D.; Kulik, B.; New, T.; Crowell, M.; Wilke, N. Liposomal MR Contrast Agents. *J. Liposome Res.* **1994**, *4*, 812−834 and references therein.

(6) A similar approach has been used for the imaging of liver: (a) Schwendener, R. A. Liposomes as Carriers for Paramagnetic Gadolinium Chelates as Organ Specific Contrast Agents for Magnetic Resonance Imaging (MRI). *J. Liposome Res.* **1994**, *4*, 837−855. (b) Kabalka, G. W.; Buonocore, E.; Hubner, K.; Davis, M.; Huang, L. Gadolinium-Labeled Liposomes Containing Paramagnetic Amphipathic Agents: Targeted MRI Contrast Agents for the Liver. *Magn. Reson. Med.* **1988**, *8*, 89−95.

(7) Paramagnetic polymerized liposomes have been previously reported; see: (a) Storrs, R. W.; Tropper, F. D.; Li, H. Y.; Song, C. K.; Kuniyoshi, J. K.; Sipkins, D. A.; Li, K. C. P.; Bednarski, M. D. Paramagnetic Polymerized Liposomes: Synthesis, Characterization, and Applications for Magnetic Resonance Imaging. *J. Am. Chem. Soc.* **1995**, *117*, 7301−7306. (b) Storrs, R. W.; Tropper, F. D.; Li, H. Y.; Song, C. K.; Sipkins, D. A.; Kuniyoshi, J. K.; Bednarski, M. D.; Strauss, H. W.; Li, K. C. Paramagnetic Polymerized Liposomes as New Recirculating MR Contrast Agents. *J. Magn. Reson. Imaging* **1995**, *5*, 719−724.

(8) Ledley, F. D. Nonviral Gene Therapy: The Promise of Genes as Pharmaceutical Products. *Hum. Gene Ther.* **1995**, *6*, 1129−1144.

(9) Kayyem, J. F.; Kumar, R. M.; Fraser, S. E.; Meade, T. J. Receptor-Targeted Co-Transport of DNA and Magnetic Resonance Contrast Agents. *Chem. Biol.* **1995**, *2*, 615−620.

(10) HPLC analysis (reverse-phase C_{18}, CH_3OH-H_2O gradient) on the derivative gadolinium−chelate indicates that partial cross-linking by the DTPA−anhydride may have occurred.

(11) Spectrum provided in the Supporting Information. For the MALDI experimental procedure, see: Cancilla, M. T.; Penn, S. G.; Carroll, J. A.; Lebrilla, C. B. Coordination of Alkali Metals to Oligosaccharide Dictates Fragmentation Behavior in Matrix Assisted Laser Desorption Ionization/Fourier Transform Mass Spectrometry. *J. Am. Chem. Soc.* **1996**, *118*, 6736−6745.

(12) Available from Avanti Polar Lipids, Alabaster, AL 35007.

(13) For reviews: (a) Felgner, P. L.; [illegible]i, Y. J.; Felgner, J. H. Advances in the Design and Application of Cytofectin Formulations. In *Nonmedical Applications of Liposomes*, 1st ed.; Lasic, D. D., Barenholz, Y., Eds.; CRC Press: Boca Raton, FL, 1996; Vol. IV, pp 43–56. (b) Behr, J.-P. Gene Transfer with Synthetic Cationic Amphiphiles: Prospects for Gene Therapy. *Bioconjugate Chem.* **1994**, *5*, 382–389.

(14) Bennett, M. J.; Aberle, A. M.; Balasubramaniam, R. P.; Malone, J. G.; Malone, R. W.; Nantz, M. H. Cationic Lipid-Mediated Gene Delivery to Murine Lung: Correlation of Lipid Hydration with in Vivo Transfection Activity. *J. Med. Chem.* **1997**, *40*, 4069–4078.

(15) Felgner, P. L.; Barenholz, Y.; Behr, J. P.; Cheng, S. H.; Cullis, P.; Huang, L.; Jessee, J. A.; Seymour, L.; Szoka, F.; Thierry, A. R.; Wagner, E.; Wu, G. Nomenclature for Synthetic Gene Delivery Systems. *Hum. Gene Ther.* **1997**, *8*, 511–512.

(16) (a) Aberle, A. [illegible]ennett, M. J.; Malone, R. W.; Nantz, M. H. The Counterion Influence on Cationic Lipid-Mediated Transfection of Plasmid DNA. *Biochim. Biophys. Acta* **1996**, *1299*, 281–283. (b) Balasubramaniam, R. P.; Bennett, M. J.; Aberle, A. M.; Malone, J. G.; Nantz, M. H.; Malone, R. W. Structural and Functional Analysis of Cationic Transfection Lipids: The Hydrophobic Domain. *Gene Ther.* **1996**, *3*, 163–172.

(17) Leventis, R.; Silvius, J. R. Interactions of Mammalian Cells with Lipid Dispersions Containing Novel Metabolizable Cationic Amphiphiles. *Biochim. Biophys. Acta* **1990**, *1023*, 124–132.

(18) Available from Promega Corp., Madison, WI 53711.

(19) Clark, R. D.; Heathcock, C. H. Preparation and Reactions of β-Chloro-α,β-unsaturated Ketones. *J. Org. Chem.* **1976**, *41*, 636–643.

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